

Low-polar extract from seed of *Cleistocalyx nervosum* var. *paniala* modulates initiation and promotion stages of chemically-induced carcinogenesis in rats

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ABSTRACT

Background: *Cleistocalyx nervosum* var. *paniala* is a local fruit mainly cultivated in the north of Thailand. Our previous study has reported that the methanol extract of *C. nervosum* seed presented antimutagenicity in a *Salmonella* mutation assay. The present study focused on the effect of a low-polar extract of *C. nervosum* seed on the early stages of diethylnitrosamine (DEN)- and dimethylhydrazine (DMH)-induced carcinogenesis in rats. **Methods:** Dried *C. nervosum* seed powder was extracted using dichloromethane. To study its effect on the initiation stage of carcinogenesis of rats, they were fed with various doses of *C. nervosum* seed extract (CSE) for 21 days. DEN injection was used to initiate hepatocarcinogenesis and partial hepatectomy was performed to amplify mutated hepatocytes resulting in micronucleated hepatocyte formation. To study the role of CSE on the promotion stage, rats were injected with DEN and DMH to induce preneoplastic lesions and the numbers of glutathione *S*-transferase placental form (GST-P) positive foci in the liver and aberrant crypt foci (ACF) in the colon were measured. This was followed by CSE administration for 10 weeks. The inhibitory mechanisms of CSE on initiation and promotion stages, including xenobiotic metabolism, cell proliferation and apoptosis, were investigated.

Results: The total phenolic content in CSE was 80.34 ± 2.29 mg gallic acid equivalents (GAE) per g of extract and 2,4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone was found to be a major flavonoid. The main terpenoids in CSE were β -selinene, α -selinene, γ -selinene and *o*-cymene while 24(*Z*)-methyl-25-homocholesterol was a major phytosterol. CSE significantly decreased the number of micronucleated hepatocytes in DEN-initiated rats and enhanced the activities of hepatic glutathione *S*-transferase and UDP-glucuronyltransferase. Furthermore, the formation of preneoplastic lesions in the liver and colon was statistically reduced by CSE. CSE also diminished cell proliferation in the liver and colon indicated by the number of PCNA positive cells. However, CSE did not alter the numbers of apoptotic hepatocytes and colonocytes in DEN- and DMH-initiated rats.

Conclusions: The dichloromethane extract of *C. nervosum* seed demonstrated chemopreventive effects on chemically-induced carcinogenesis in both initiation and promotion stages in rats. The inhibitory mechanism might be involved in the modulation of hepatic detoxifying enzymes and suppression of hepatocyte and colonocyte proliferation.

Abbreviations: 4-NP, 4-nitrophenol; ACF, aberrant crypt foci; CDNB, 1-chloro-2,4-dinitrobenzene; CPR, NADPH-cytochrome P450 reductase; DAB, 3,3'-diaminobenzidine; DAPI, 4',6'-diamidino-2-phenylindole; DCPIP, 2,6-dichlorophenol-indophenol; DEN, diethylnitrosamine; DMH, 1,2-dimethylhydrazine; GST, glutathione *S*-transferase; GST-P, glutathione *S*-transferase placental form; HRP, horseradish peroxidase; NQO-1, NADPH-quinone oxidoreductase; NSS, normal saline solution; PCNA, proliferating cell nuclear antigen; UGT, UDP-glucuronyltransferase.

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1. Introduction

Use of phytochemicals is currently regarded as one of the important methods for cancer prevention. Numerous studies have been focused on their protective roles in the prevention of either the initiation or promotion stages of carcinogenesis [1]. Cancer chemopreventive agents can be classified into two types depending on the mode of action: either blocking agents or suppressing agents [2]. Blocking agents can prevent the initiation stage by modulation of detoxification systems. Moreover, these agents also act as reactive species scavengers and inducers of DNA repair [2,3]. Suppressing agents inhibit the evolution of the neoplastic process in either the promotion or the progression stages. These agents can reduce initiated cell proliferation, restore apoptosis to normal

levels, decrease inflammation and also scavenge reactive species [2,4].

Fruits, vegetables, spices and cereals are major sources of promising cancer chemopreventive agents including phenolics, flavonoids, alkaloids and terpenoids [5,6]. Many studies have also shown some natural compounds from non-edible plants or inedible parts of plants are also an attractive source of chemopreventive agents [7]. Apple waste extract was found to modulate colon carcinogenesis *in vitro* [8]. Litchi fruit pericarp and seed extract exhibited potential anticancer activity against hepatocellular carcinoma *in vitro* and *in vivo* [9]. In addition, the tocotrienol-rich fraction of grape seeds was found to have antioxidant and antiproliferative activities [10].

Cleistocalyx nervosum var. *paniala* is a local fruit in some areas of Southeast Asia including northern Thailand. Our previous studies reported that the methanol extract of *C. nervosum* flesh showed antioxidant [11,12] and antimutagenic activities [13]. *C. nervosum* seed, a waste from processing products, contains high phenolic and flavonoid contents which show antibacterial activity against *Propionibacterium acnes* and *Staphylococcus aureus*. Furthermore, the hydrophilic parts of this seed have no genotoxicity in both *in vitro* and *in vivo* models and also presented antigenotoxicity in a bacterial mutation assay [13]. However, the effect of the lipophilic part of the *C. nervosum* seed on cancer prevention has not been observed. Thus, the present study aimed to investigate the antimutagenicity and anticarcinogenicity of the dichloromethane extract of the *C. nervosum* seed in animal models.

2. Materials and methods

2.1. Chemicals

1,2-Dimethylhydrazine (DMH) was obtained from Oriental Yeast Co., Ltd. (Tokyo, Japan). Diethylnitrosamine (DEN) and diaminobenzidine (DAB) were purchased from Sigma-Aldrich Corp. (St. Louis, MO, USA). Primary rabbit polyclonal antibodies against rat GST-P and monoclonal mouse anti-rat PCNA were obtained from MBL (Nagoya, Japan) and Biolegend (San Diego, CA, USA), respectively. The Apoptag detection kit was purchased from Merck Millipore (Burlington, MA, USA). The EnVision Doublestain system was obtained from DAKO (Agilent, Santa Clara, CA, USA). The avidin-biotin-horseradish peroxidase complex (ABC) kit and Vectastain Elite ABC kit (Universal) were purchased from Vector Laboratories Inc. (Burlingame, CA, USA).

2.2. Extraction and determination of some phytochemicals

The seeds of *C. nervosum* (voucher number: QGB 7290, QGB 17340, QGB 25139) were separated from ripe fruits and dried at 40 °C with a hot air dryer. The dried seeds were ground into powder and soaked in dichloromethane at room temperature for three days. A brown sticky extract was obtained after evaporation and lyophilization.

The total phenolic content of *C. nervosum* seed extract (CSE) was examined using the Folin-Ciocalteu technique as previously described [13]. The content was determined using gallic acid for calibration and was expressed as mg of gallic acid equivalents (GAE) per g of extract.

The content of 2,4'-dihydroxy-6-methoxy-3,5'-dimethylchalcone (DMC), a pure compound isolated from *C. nervosum* seed, in crude extract, was determined using high performance liquid chromatography (HPLC) according to Portet et al. [14] with some modifications. HPLC was performed with the Agilent Technologies 1200 infinity series system (Agilent, Santa Clara, CA, USA). The separation was achieved on a Zorbax Eclipse Plus C18 column (4.6 × 150 mm, inner diameter 5 µm) (Agilent, Santa Clara, CA, USA). The extract was eluted with a gradient elution of mobile phase A (water) and B (methanol). A gradient program was set as follows: 60 %B – 100 %B (20 min), 100 %B isocratic (5 min), 60 %B (2 min) and 60 %B isocratic (5 min). DMC was monitored at 340 nm at a flow rate of 1 mL/min.

The profile of non-polar bioactive compounds in CSE was examined using gas chromatography – mass spectrometry (GC-MS) (Model 6890 N/5973, Agilent, Santa Clara, CA, USA). The GC-MS was carried out using an Rxi-5 ms column (30 m

(L) × 0.25 mm (i.d.) × 0.25 µm film thickness; Restek Corporation, Bellefonte, United States). Helium was used as a carrier gas at a flow of 1 mL/min. The GC oven temperature was programmed from 45 °C to 280 °C at a ramp rate of 4 °C/min. The sample injection volume was 1 µL at the injector temperature of 250 °C in split mode (1:50). The MS spectra were analysed in the electron impact (EI) mode at 70 eV and the acquisition mode scan ranged from 29 to 550 amu [15]. The ion source and quadrupole temperatures were 230 °C and 150 °C, respectively. The bioactive compounds were identified by matching their mass spectra with reference spectra in NIST libraries from the database.

2.3. Animals

Male Wistar rats, three weeks old, weighing approximately 50–80 g, were obtained from the National Laboratory Animal Center (Nakhon Pathom, Thailand). They were housed under standard environmental conditions of temperature at 25 °C under a 12 h dark-light cycle and allowed free access to drinking water and a pelleted diet. An experimental protocol was approved by The Animal Ethics Committee of the Faculty of Medicine, Chiang Mai University (Approval number: 35/2554).

2.4. Mutagenicity and antimutagenicity

Rats were divided into six groups as shown in Fig. 1A. Groups 1 and 3 received 5% Tween-80 as a vehicle control. Groups 4 and 5 orally received 100 and 250 mg/kg body weight (bw) of CSE, respectively. Groups 2 and 6 were treated with 500 mg/kg bw of CSE for 21 days. Rats in groups 3–6 were injected with 30 mg/kg bw of DEN by intraperitoneum twice on days 15 and 18 of an experiment to induce micronucleated hepatocytes. All rats were 2/3 partially hepatectomized to amplify initiated hepatocytes on day 22. After four days, single hepatocytes were isolated by the two-step collagenase perfusion method according to Inboot et al. [13]. The hepatocytes were stained with 4', 6-diamidino-2-phenylindole (DAPI) and counted under a fluorescent microscope. The numbers of micronucleated hepatocytes and mitotic cells were scored based on analysis of 2000 hepatocytes of each animal. The criteria for micronucleated hepatocytes followed the method of Nilnumkhum et al. [16].

2.5. Measurement of some xenobiotic metabolizing enzymes

The cytosolic and microsomal fractions of the liver were prepared by ultracentrifugation. The amount of protein was quantified by the Lowry method. The NADPH-cytochrome P450 reductase (CPR) activity was measured according to Punvittayagul et al. [17] using cytochrome c as an electron acceptor. The enzyme activity was determined at 550 nm and calculated using extinction coefficient 21 M⁻¹ cm⁻¹. Enzyme activity was expressed as U/mg protein. Glutathione S-transferase (GST) activity was analysed by the method of Sankam et al. [18] using 1-chloro-2, 4-dinitrobenzene (CDNB) as a substrate with a slight modification.

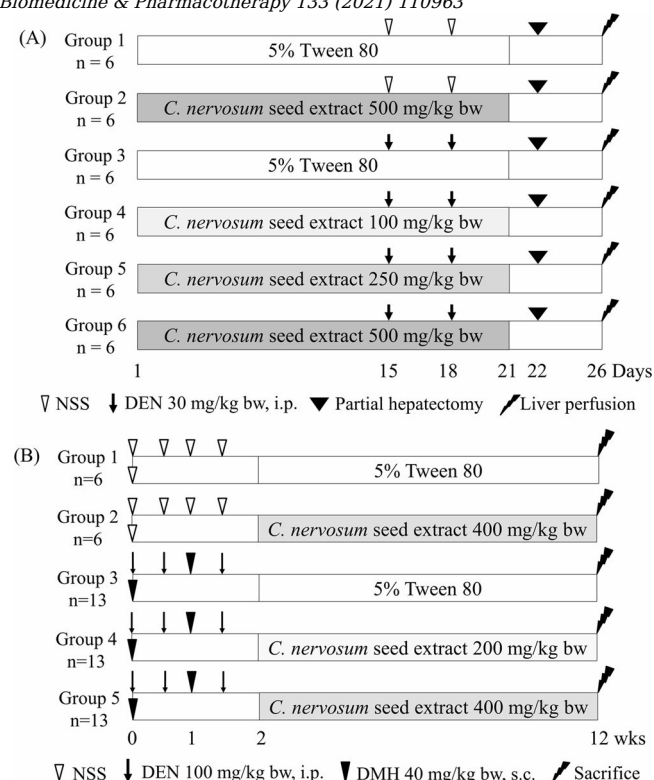


Fig. 1. The protocol for the study of the chemopreventive effect of the dichloromethane extract of *Cleistocalyx nervosum* var. *paniala* seed in rats. (A) Mutagenicity and antimutagenicity against diethylnitrosamine in liver. (B) Carcinogenicity and anticarcinogenicity against diethylnitrosamine and dimethylhydrazine in liver and colon.

The change in absorbance was recorded at 340 nm. The GST activity was shown as nmol CDNB conjugate formed/min/mg protein using an extinction coefficient of $9.6 \text{ M}^{-1} \text{ cm}^{-1}$. NAD(P)H-quinone oxidoreductase (NQO-1) activity was determined using 2,6-dichlorophenolindophenol (DCPIP) as an electron acceptor [18]. The disappearance of DCPIP substrate was measured at 600 nm. The NQO-1 activity was expressed as pmol of reduced DCPIP/min/mg protein using an extinction coefficient of $2.1 \times 10^4 \text{ M}^{-1} \text{ cm}^{-1}$. UDP-glucuronyltransferase (UGT) activity was studied using a modified method of Chariyakornkul et al. [19]. The substrate used was 4-nitrophenol (4-NP). The absorbance of conjugated 4-NP was measured at 405 nm. The UGT activity was expressed as pmol 4-NP conjugate formed/min/mg protein.

2.6. Carcinogenicity and anticarcinogenicity

Male Wistar rats were randomly divided into five groups (Fig. 1B). Groups 1 and 2 were injected with normal saline solution (NSS) (4 mL/kg bw), while groups 3–5 were intraperitoneally injected with 100 mg/kg bw of DEN three times, on days 0, 4 and 11 of an experiment to initiate hepatocarcinogenesis and 40 mg/kg bw of DMH by subcutaneous injection twice on days 0 and 7 of an experiment to initiate colorectal carcinogenesis [20]. After the third DEN injection for 3 days, groups 1 and 3 were fed by 5% Tween-80 as a vehicle control. Group 4 orally received 200 mg/kg bw of CSE while groups 2 and 5 received 400 mg/kg bw of CSE, for 10 weeks. At the end of the experiment, rats were sacrificed under light anesthesia. The liver was cut into three

pieces from each lobe and the large intestine was cut into three parts: proximal and distal parts of the colon as well as the rectum. The specimens were fixed with 10 % neutral buffered formalin and embedded in a paraffin block. The colonic tissues were stained with methylene blue. The number of aberrant crypt foci (ACF) was counted under light microscope and expressed as frequency (number of crypts per colon) and multiplicity (number of crypts per focus) of ACF.

2.7. Immunohistochemical staining

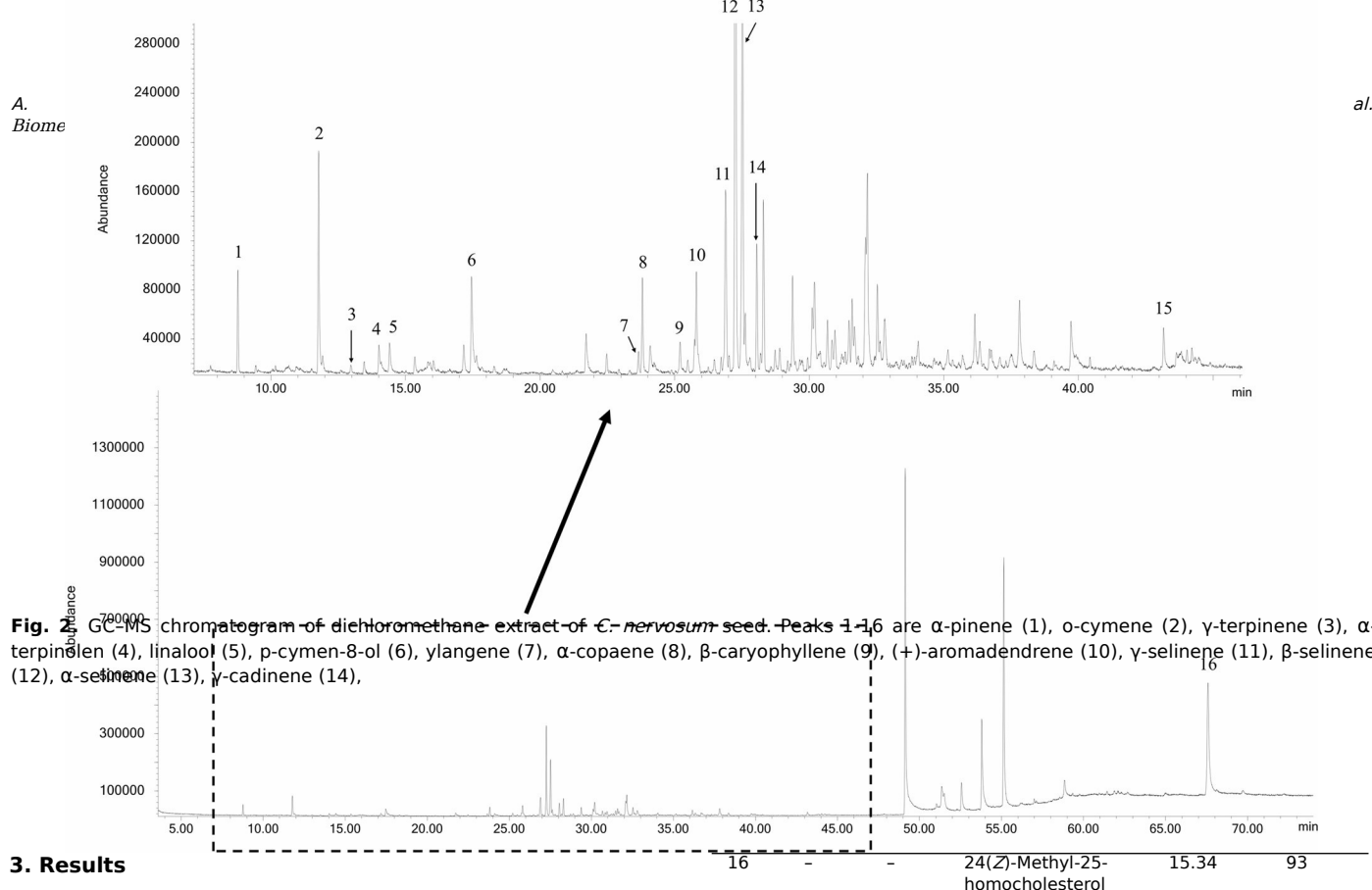
Paraffin-embedded liver tissues were cut into 4 μm thick sections. The immunohistochemistry of glutathione S-transferase placental form (GST-P) in the liver was determined by the method of Puatanachokchai et al. [21]. Liver sections were incubated with rabbit polyclonal anti-rat GST-P antibody for 2 h and their colour was developed with 3,3'-diaminobenzidine (DAB). The number of GST-P positive foci was counted using a light microscope and reported as the number of GST-P per cm^2 of liver area.

Double staining of proliferating cell nuclear antigen (PCNA) or apoptotic hepatocytes in GST-P positive foci and surrounding areas were performed using the EnVision system. The liver sections were incubated with monoclonal mouse anti-rat PCNA antibody for 2 h. After that, polymer/horseradish peroxidase (HRP) was applied and the colour was generated with DAB. Moreover, the apoptotic cell detection of the liver was analysed as described by Thumvijit et al. [22]. After their colour was developed with DAB, all slides were incubated with double-stain block reagent followed by incubation with the anti-rabbit GST-P antibody for 1 h. The secondary antibodies against mouse and rabbit immunoglobulins, Rabbit/Mouse (LINK), were applied to the slides, followed by incubation with alkaline phosphatase. The reaction product was visualized using permanent red working solution. Lastly, the sections were counterstained with haematoxylin and slides were mounted using mounting solution. The numbers of brown staining PCNA or TUNEL positive cells were counted both inside and surrounding GST-P positive foci.

The immunohistochemistry of the PCNA positive cells in colonic tissue were analysed. The cross-section paraffin-embedded colonic tissues were deparaffinized and immersed in 10 mM citrate buffer (pH 6.0) in a pressure cooker to activate antigenicity. Then, the sections were incubated with monoclonal mouse anti-rat PCNA antibodies, then incubated with biotinylated antibodies and this was followed by using an Elite avidin-biotin complex kit. The Apoptag detection kit was used to measure apoptotic cells in tissues. The processes were carried out by following kit instructions. The resulting colour was developed by DAB reaction. The numbers of PCNA and apoptotic cells were determined under a light microscope and reported as the number of PCNA positive cell or apoptotic cells per rat.

2.8. Statistical analysis

Data are reported as mean $\pm$ SD. The significance of difference between group was determined by one-way analysis of variance using a post hoc least-significant difference test. Values of $P < 0.05$ were considered to be significant.



3. Results

3.1. Chemical constituents in CSE

One hundred gram of dried *C. nervosum* seed obtained 1.22 g of crude dichloromethane extract. A gram of the extract contained 80.34 ± 2.29 mg GAE of total phenolic compounds measured by spectrophotometric method. The content of DMC in a crude extract was 62.40 ± 1.71 μ g/mg of extract analysed by HPLC analysis.

The MS fingerprint of bioactive compounds in CSE is presented in Fig. 2. A total of 71 bioactive compounds were identified. These included 54 unknown compounds and 16 identified compounds which were phytosterols and terpenoids including sesquiterpenes and monoterpenes (Table 1). The major identified compounds in CSE were 24(Z)-methyl-25-homocholesterol (15.34 %), β -selinene (4.27 %), α -selinene (2.87 %), γ -selinene (1.15 %) and o-cymene (1.09 %). phytol isomer (15) and 24(Z)-methyl-25-homocholesterol (16).

Table 1

Identified compounds and percentage relative contents of chemical components in seed of *C. nervosum*.

Peak No.	LRlcal ^a	LRlit ^b	Identified compound	Relative content (%)	Quality (%)
1	933	932	α -Pinene	0.42	97
2	1024	1022	o-Cymene	1.09	97
3	1058	1054	γ -Terpinene	0.06	94
4	1088	1086	α -Terpinolene	0.22	94
5	1101	1095	Linalool p-Cymen-8-ol	0.20	90
6	1187	1187 ^c		0.68	98
7	1373	1373	α -Ylangene	0.11	97
8	1378	1374	α -Copaene	0.47	99
9	1422	1417	β -Caryophyllene (+)-	0.16	99
10	1478	1472 ^c	Aromadendrene γ -	0.65	96
11	1479	1479 ^c	Selinene	1.15	99
12	1489	1489	β -Selinene	4.27	99
13	1498	1498	α -Selinene	2.87	99
14	1517	1513	γ -Cadinene	0.62	98
15	2114	2114 ^c	Phytol	0.24	97

^aLinear retention indices as determined on a Rxi-5 ms column using the homologous series of n- alkanes (C₈-C₃₀); ^b Linear retention indices values from Adams, 2007 [23]; ^c Data not referred in Adams, 2007 and thus obtained from National Institute of Standards and Technology (NIST).

3.2. Effect of CSE on micronucleus formation in liver of rats

The body weight and water and food consumption of CSE treated rats were not significantly different from a vehicle control group (data not shown). The administration of 500 mg/kg bw of CSE for 21 days did not affect the number of micronucleated hepatocytes and mitotic index when compared with a control group (Fig. 3). DEN significantly increased the number of micronucleus formation in rat liver compared to a control group. Interestingly, oral administration of CSE statistically reduced the frequencies of micronucleus formation in liver of DEN- initiated rats in a dose-dependent manner. Furthermore, it also had no impact on the mitotic index.

3.3. Effect of CSE on xenobiotic metabolizing enzymes in rat liver

The treatment of CSE significantly increased the activities of GST and UGT compared to a DEN alone group. The CSE did not affect the activities of NADPH-cytochrome P450 reductase and NADPH-quinone oxidoreductase-1 (Table 2).

3.4. Effect of CSE on preneoplastic lesions in liver and colon of rats

During the experiment, no difference was noted in food and water consumption between treated and control groups. The body and some vital organ weights and serum liver function enzyme level of rats are illustrated in Table 3. When DEN and

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DMH were injected into rats, their body weight at the end of experiment significantly decreased in together with increased serum AST and ALT levels and the relative liver and spleen weights compared to a control group. Oral administration of CSE (groups 4 and 5) significantly increased the relative liver and kidney weights compared to carcinogen-treated rats. In addition, the CSE- and NSS-treated rats had statistically reduced final body weights while they did not show the change of relative organ weights compared with a

Table 3

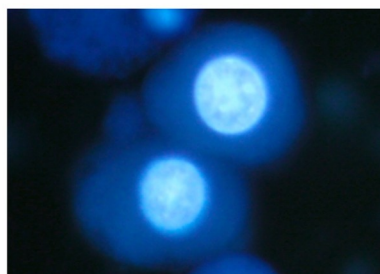
negative control group.

CSE neither induced hepatic GST-P positive foci nor colonic ACF (Table 4). DEN- and DMH-initiated rats significantly induced GST-P positive foci and ACF formation when compared with a negative control (group 1). The administration of CSE at a dose of 200 mg/kg bw significantly decreased the number of hepatic GST-P positive foci in DEN- and DMH-treated rats but did not reduce the size of positive foci. Furthermore, the CSE

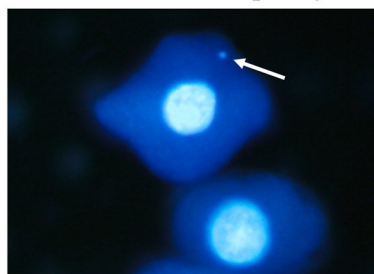
Fig. 3. Effect of dichloromethane extract of *Cleistocalyx nervosum* var. *paniala* seed on liver micronucleus formation in rats. (A) Normal hepatocytes and micronucleated hepatocyte. (B) The number of micronucleated hepatocytes.

Values expressed as mean $\pm$ SD., CSE: *nervosum* seed extract. * significantly different from negative control group, $p < 0.05$. # significantly different from DEN control group, $p < 0.05$.

(A) Normal hepatocytes



Micronucleated hepatocyte



(B)

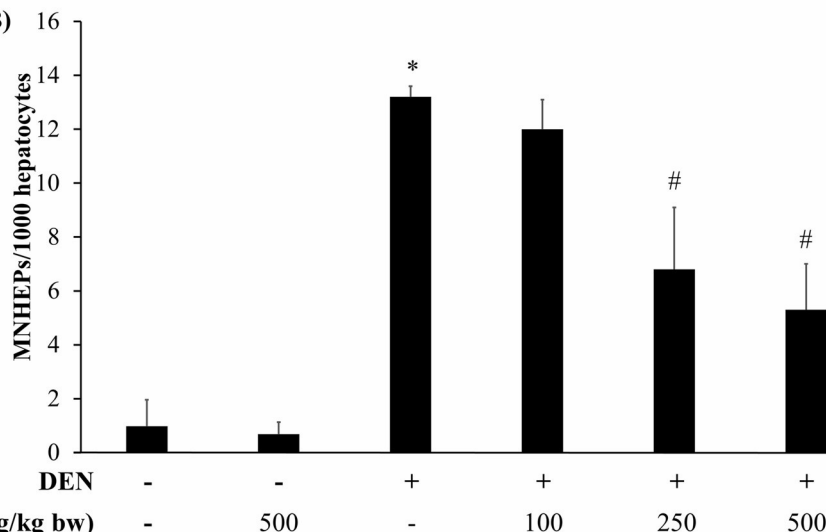


Table 2

Effect of *C. nervosum* seed extract on the activities of some hepatic xenobiotic metabolizing enzymes in diethylnitrosamine initiated rats.

Group	Treatment	CPR (U/mg protein)	NQO-1 (U/mg protein)	GST (U/mg protein)	UGT (nmol/min/ mg protein)
1	DEN + 5% Tween 80	0.15 $\pm$ 0.03	5.82 $\pm$ 3.01	37.11 $\pm$ 12.31	3.18 $\pm$ 0.31
2	DEN + 100 mg/kg bw CSE	0.14 $\pm$ 0.06	6.17 $\pm$ 3.57	52.73 $\pm$ 11.62	6.04 $\pm$ 1.24*
3	DEN + 250 mg/kg bw CSE	0.14 $\pm$ 0.03	5.90 $\pm$ 2.25	59.44 $\pm$ 9.78*	6.45 $\pm$ 1.46*
4	DEN + 500 mg/kg bw CSE	0.16 $\pm$ 0.04	11.55 $\pm$ 8.00	82.31 $\pm$ 13.76*	7.22 $\pm$ 9.84*

The data are expressed as a Mean $\pm$ SD. * $p < 0.05$ compared with group 1. CSE: *C. nervosum* seed extract, CPR: cytochrome P450 reductase, DEN: diethylnitrosamine, GST: glutathione *S*-transferase, NQO-1: NAD(P)H quinone oxidoreductase-1, UGT: UDP-glucuronyltransferase.

Body and some vital organ weights and serum liver function enzyme levels in rats.

Groups	Treatments	Initial body weight (g)	Final body weight (g)	Relative organ weight (%)	
				Liver	
					AST (U/L) ALT (U/L)

statistically decreased the frequency of ACF but did not affect crypt multiplicity in the colon of DEN- and DMH-initiated rats.

3.5. Effect of CSE on proliferation and apoptosis in liver and colon

Due to CSE significantly reducing preneoplastic lesions in both the liver and colon of carcinogen-treated rats, the possible mechanisms of cellular proliferation and apoptosis of the extract were examined. The numbers of positive PCNA and apoptotic cells in GST-P positive foci and surrounding area of rat liver are shown in Table 5. Treatment with DEN and DMH significantly increased the number of PCNA positive

									Spleen	Kidney 1	NSS
+ 5% Tween 80	164 ± 11	489 ± 17	81 ± 16	25 ± 3	2.99 ± 0.18	0.17 ± 0.02	0.49 ± 0.03	2	NSS + 400 mg/kg bw CSE		
	164 ± 7	432 ± 23*	86 ± 15	28 ± 4	3.25 ± 0.10	0.16 ± 0.02	0.50 ± 0.02	3	DEN & DMH + 5% Tween 80	164	
± 10	410 ± 19*	125 ± 26*	51 ± 15*	3.28 ± 0.24*	0.23 ± 0.03*	0.48 ± 0.04					
4	DEN & DMH + 200 mg/kg bw CSE			164 ± 9	416 ± 24	107 ± 23	65 ± 11	3.59 ± 0.18*	0.23 ± 0.05	0.56 ± 0.06*	5
	DEN & DMH + 400 mg/kg bw CSE			164 ± 11	411 ± 38	107 ± 29	54 ± 21	3.55 ± 0.29*	0.22 ± 0.07	0.55 ± 0.07*	

The data are expressed as a Mean ± SD. * $p < 0.05$ compared with group 1. # $p < 0.05$ compared with group 3.

ALT: alanine aminotransferase, AST: aspartate aminotransferase, CSE: *C. nervosum* seed extract, DEN: diethylnitrosamine, DMH: 1,2-dimethylhydrazine.

Table 4

Effect of *C. nervosum* seed extract on preneoplastic lesion formation of diethylnitrosamine and 1,2-dimethylhydrazine initiated rats.

Groups	Treatment	GST-P positive foci				Aberrant crypt foci			
		Foci number (foci/ 2mm)		Frequency (crypt/rat)		Multiplicty (crypt/focus)		Colon	
1	NSS + 5% Tween 80	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0	0.0 ± 0.0
3	DEN & DMH + 5% Tween 80	10.4 ± 3.8*	2.5 ± 2.4	243.3 ± 44.1*	17.2 ± 7.2*	4.5 ± 0.8*	3.2 ± 0.5*		
4	DEN & DMH + 200 mg/kg bw CSE	6.4 ± 2.1*	2.5 ± 1.8	184.0 ± 37.7*	14.9 ± 6.6	4.6 ± 0.9	3.2 ± 0.5		
5	DEN & DMH + 400 mg/kg bw CSE	12.1 ± 2.5	2.2 ± 1.4	213.6 ± 55.4	28.0 ± 21.1	4.9 ± 1.0	3.2 ± 1.2		

The data are expressed as a Mean ± SD. * $p < 0.05$ compared with group 1. # $p < 0.05$ compared with group 3.

CSE: *C. nervosum* seed extract, DEN: diethylnitrosamine, DMH: 1,2-dimethylhydrazine, GST-P: glutathione S-transferase placental form.

Table 5

Effect of *C. nervosum* seed extract on cellular proliferation and apoptosis in preneoplastic lesions and their surrounding areas in rat liver.

Groups	Treatment	No. of PCNA positive cell/ 1000 hepatocytes		No. of apoptotic cell/1000 hepatocytes	
		GST-P positive area foci	Surrounding positive area foci	GST-P positive area foci	Surrounding positive area foci
1	NSS + 5% Tween 80	0.0 ± 0.0	31.8 ± 5.9	0.0 ± 0.0	11.8 ± 3.1
2	NSS + 400 CSE	0.0 ± 0.0	22.7 ± 2.8	0.0 ± 0.0	9.1 ± 2.1
3	DEN & DMH + 5% Tween 80	165.3 ± 29.1*	55.1 ± 11.3*	13.4 ± 2.7*	13.3 ± 2.7
4	DEN & DMH + 200 mg/kg bw CSE	85.5 ± 17.7*	44.8 ± 11.6	12.1 ± 0.8	10.9 ± 4.2
5	DEN & DMH + 400 2.1 mg/kg bw mg/kg bw CSE	99.6 ± 22.0*	35.4 ± 6.5*	6.5 ± 1.5*	7.1 ± 1.1*

The data are expressed as a Mean ± SD. * $p < 0.05$ compared with group 1. # $p < 0.05$ compared with group 3.

CSE: *C. nervosum* seed extract, DEN: diethylnitrosamine, DMH: 1,2-dimethylhydrazine, GST-P: glutathione S-transferase placental form, PCNA: proliferating cell nuclear antigen.

hepatocytes in both GST-P positive foci and the surrounding area, while they increased the number of apoptotic cells in only GST-P positive foci. Administration of CSE at 200 mg/kg bw significantly reduced the number of PCNA only in GST-P

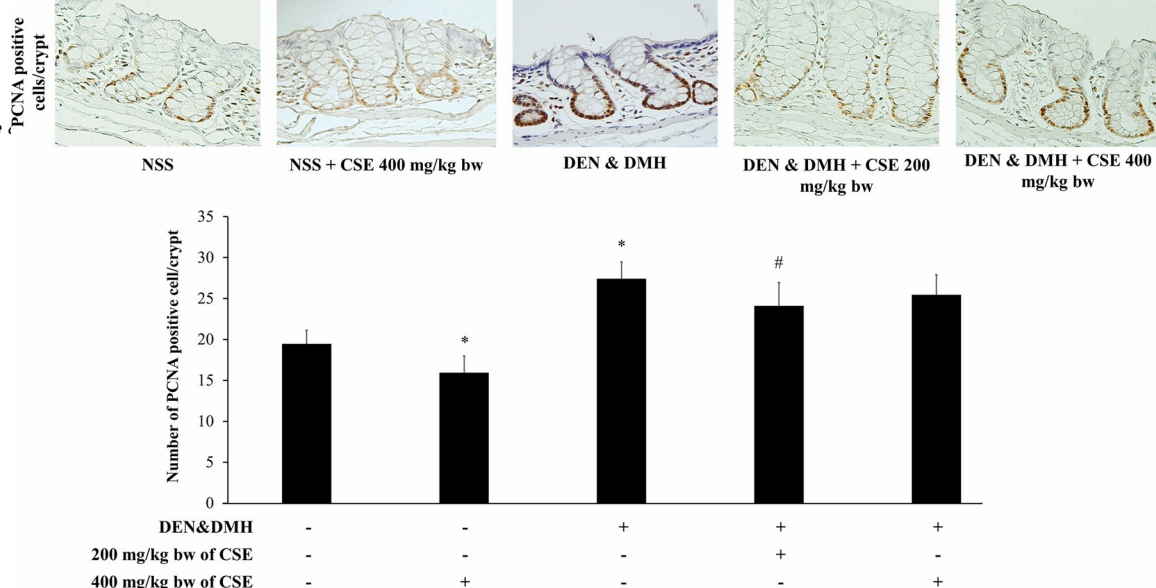
positive foci and did not affect apoptotic cells in DEN- and DMH-initiated rats. CSE at 400 mg/kg bw significantly decreased the numbers of PCNA and apoptotic cell in both GST-P positive foci and surrounding tissues.

The number of colonic PCNA positive cells statistically increased in carcinogen-treated rats compared with a control group. A low dose of CSE significantly lowered the number of PCNA positive cells in colon of DEN and DMH initiated rats (Fig. 4). Furthermore, the CSE did not influence cellular apoptosis in the colon of carcinogens treated rats (Fig. 5). However, a high dose of CSE significantly decreased the numbers of PCNA positive cells and apoptotic cells compared with the control group.

4. Discussion

Several studies have shown that non-edible parts of fruits and vegetables, agricultural co-products, are attractive sources of cancer chemopreventive compounds [7]. This study found that the dichloromethane extract of *C. nervosum* seed exhibited antimutagenicity and anticarcinogenicity in both early stages of chemically - induced carcinogenesis in rats.

Xenobiotic metabolism of particular carcinogens plays an important role in the initiation stage of chemical carcinogenesis. The resulting metabolites may adduct to DNA leading to DNA mutated cells after failure of DNA repair [24]. Micronucleus formation in targeted cells has been widely used as a biomarker of genotoxic consequence including DNA damage and chromosome aberration [25]. We found the administration of the dichloromethane extract of *C. nervosum* seed significantly decreased the number of micronucleated hepatocytes initiated by DEN in a dose-dependent manner. It also increased the activities of GST and UGT in the liver of DEN-initiated rats. Several studies have shown that phytochemicals could modulate phase II enzyme activities [3]. Green and white tea extracts protected benzo(a)pyrene (BaP) induced DNA adduct in liver and lung through elevation of the activities of GST and NADPH-quinone reductase (QR) [26]. Moreover, purple rice bran extract presented antigenotoxicity



in aflatoxin B₁-initiated rats by induction of the activities of phase II xenobiotic metabolizing enzymes such as GST and UGT [27]. These results suggest that CSE might inhibit liver micronucleus formation by induction of detoxifying enzyme systems.

Moreover, CSE has shown anticarcinogenic properties in DEN and DMH induced carcinogenesis in rats. DEN is a hepatocarcinogen metabolized by CYP2E1 in the liver to ultimate carcinogen and interacts with DNA resulting in DNA mutation and carcinogenesis [28]. DMH, a potent colon carcinogen, is also metabolized by CYP2E1 resulting in the production of the methyldiazonium ion which can alkylate DNA in the colon including O⁶-methyl-deoxyguanosine and N⁷-methyl-deoxyguanosine leading to colon carcinogenesis [29]. Using the combined treatment of DEN and DMH could induce preneoplastic lesions in both the liver and colorectal tissues of rats [20]. The administration of CSE during the promotion stage of DEN- and DMH-induced carcinogenesis significantly decreased the formation of hepatic GST-P positive foci and colonic ACF, which are commonly used as the end point markers of early stages of hepatocarcinogenesis and colorectal carcinogenesis, respectively. CSE also did not induce these biomarkers in rats indicating no carcinogenicity. Remarkably, the administration of CSE at 200 mg/kg bw significantly decreased preneoplastic lesion formation in the liver and colon of carcinogens induced rats, but the inhibitory outcome was not found in higher dose administration. CSE at low dose selectively reduced amount of cell proliferation in preneoplastic lesions but did not affect apoptosis in hepatocytes and colonocytes resulting in anticarcinogenicity in the liver and colon. On the other hand, CSE at high dose reduced cell proliferation and suppressed apoptosis in pan-hepatic tissues. From this evidence, anticarcinogenicity of CSE

at a dosage of 400 mg/kg bw could not be observed in DEN- and DMH-induced rats. Likewise, it is implied that treatment with a large amount of CSE might cause toxicity. The cancer chemopreventive activity of CSE seems to be attributed to the combined effect of its ingredients, rather than to specific compounds. Some phenolic compounds had biphasic effects on cellular regulation [30,31]. The modulation on cell proliferation was notably dose dependent. At high concentration, some phenolic compounds were shown to have a direct toxic effect [32].

Some phytochemicals including curcumin and sulforaphane [33],

Fig. 4. Effect of *C. nervosum* seed extract on cell proliferation in rat colon.

Values expressed as mean ± SD., CSE: *C. nervosum* seed extract.

* Significantly different from negative control group, $p < 0.05$. #

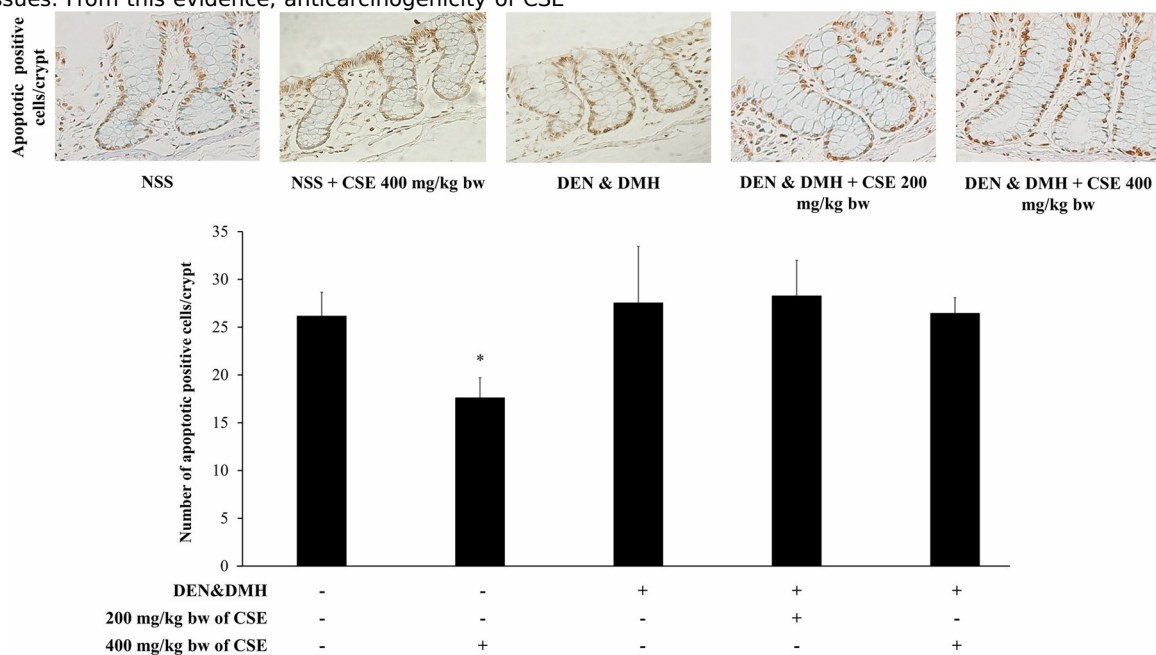
Significantly different from positive control group, $p < 0.05$.

Fig. 5. Effect of *C. nervosum* seed extract on cell apoptosis in rat colon.

Values expressed as mean ± SD., CSE: *C. nervosum* seed extract.

* Significantly different from negative control group, $p < 0.05$.

possess both blocking and suppressing effects. Chalcones are one member of the flavonoids which exhibit numerous biological benefits such as antioxidant, antibacterial, anti-inflammatory, anticancer, anti-mitotic, cytotoxic and immunosuppressive activities [34]. Notably, DMC, a promising chalcone found in *Cleistocalyx operculatus*, was also found in CSE. DMC could inhibit the growth of several human cancer cell line [35,36]. Moreover, DMC showed a potential hepatoprotective effect through reduction of oxidative stress



[37]. It also suppressed colorectal carcinoma HCT116 and LOVO cell proliferation and induced autophagy [38]. Among lipophilic phytochemicals, terpenoids are a large and diverse group. They are the main components of essential oils of several plants and flowers [39]. β -Selinene is one of the essential oils from *Leonurus sibiricus* L. and *Callicarpa macrophylla* that presented antibacterial, anti-inflammatory, antioxidant and antiproliferative properties [40,41]. α -Selinene found in essential oils from *Teucrium yemense* exhibited cytotoxicity against human colorectal adenocarcinoma cell lines (HT-29) and human breast adenocarcinoma cell lines (MCF-7 and MDA-MB-231) [42]. α -Cymene, identified from volatile oil of *Satureja hortensis* L., presented anticancer activity on A375 human melanoma cells and B16 mouse melanoma 4A5 cells [43]. Phytosterols are commonly found in vegetable oil, seed, cereal and legumes which showed several biological activities such as anti-inflammatory and anticancer activities [44]. Much research has found 24(Z)-methyl-25-homocholesterol in the lipophilic extract of various plants such as *Piper betle* [45], *Allamanda* species [46] and red algae (Rhodophyta) [47]. Likewise, this sterol was also found in *Dryopteris crassirhizoma* that presented an anticancer effect in PC3-MM2 human prostate cancer cell lines [48]. Based on these results, CSE might act as both a blocking agent and a suppressing agent on carcinogenesis. DMC, β -selinene, α -selinene, α -cymene and 24(Z)-methyl-25-homocholesterol might be the functional ingredients in CSE.

5. Conclusions

The dichloromethane extract of *Cleistocalyx nervosum* var. *paniala* seeds containing various low polar phytochemicals including chalcone flavonoids, terpenoids and phytosterols demonstrated a chemopreventive effect on both the initiation and promotion stages of chemically induced early stages of carcinogenesis. The inhibitory mechanism might be due to the improvement of detoxifying enzymes and suppression of cell proliferation leading to reduction of preneoplastic lesions in rat liver and colon.

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Declaration of Competing Interest

The authors declare that they have no conflict of interest.

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References

- [1] V. Kaur, M. Kumar, A. Kumar, K. Kaur, V.S. Dhillon, S. Kaur, Pharmacotherapeutic potential of phytochemicals: implications in cancer chemoprevention and future perspectives, Biomed. Pharmacother. 97 (2018) 564–586.

- [2] I.A. Siddiqui, V. Sanna, N. Ahmad, M. Sechi, H. Mukhtar, Resveratrol nanoformulation for cancer prevention and therapy, Ann. N. Y. Acad. Sci. 1348 (1) (2015) 20–31.
- [3] M.T. Lee, W.C. Lin, B. Yu, T.T. Lee, Antioxidant capacity of phytochemicals and their potential effects on oxidative status in animals - A review, Asian-Australas. J. Anim. Sci. 30 (3) (2017) 299–308.
- [4] L. Budisan, D. Gulei, O.M. Zanoaga, A.I. Irimie, C. Sergiu, C. Braicu, C.D. Gherman, I. Berindan-Neagoe, Dietary intervention by phytochemicals and their role in modulating coding and non-coding genes in cancer, Int. J. Mol. Sci. 18 (6) (2017) 1178.
- [5] P.G. Anantharaju, P.C. Gowda, M.G. Vimalambike, S.V. Madhunapantula, An overview on the role of dietary phenolics for the treatment of cancers, Nutr. J. 15 (1) (2016) 99.
- [6] G. Guerriero, R. Berni, J.A. Munoz-Sanchez, F. Apone, E.M. Abdel-Salam, A. A. Qahtan, A.A. Alatar, C. Cantini, G. Cai, J.F. Hausman, K.S. Siddiqui, S.M. T. Hernandez-Sotomayor, M. Faisal, Production of plant secondary metabolites: examples, tips and suggestions for biotechnologists, Genes 9 (6) (2018) 309.
- [7] S. Ji, B. Orlikova, M. Diederich, Non-edible plants as an attractive source of compounds with chemopreventive potential, J. Cancer Prev. 19 (1) (2014) 1–6. [8] M.J. McCann, C.I. Gill, J.R. Rao, W.C. McRoberts, P. Hughes, R. McEntee, I. R. Rowland, Anti-cancer properties of phenolics from apple waste on colon carcinogenesis *in vitro*, Food Chem. Toxicol. 45 (7) (2007) 1224–1230.
- [9] S. Emanuele, M. Lauricella, G. Calvaruso, A. D'Anneo, M. Giuliano, *Litchi chinensis* as a functional food and a source of antitumor compounds: an overview and a description of biochemical pathways, Nutrients 9 (9) (2017) 992.
- [10] Y. Choi, J. Lee, Antioxidant and antiproliferative properties of a tocotrienol-rich fraction from grape seeds, Food Chem. 114 (4) (2009) 1386–1390.
- [11] S. Taya, C. Punvittayagul, W. Inboot, S. Fukushima, R. Wongpoomchai, *Cleistocalyx nervosum* extract ameliorates chemical-induced oxidative stress in early stages of rat hepatocarcinogenesis, Asian Pac. J. Cancer Prev. 15 (6) (2014) 2825–2830.
- [12] W. Thuschana, S. Chanasakaow, S. Ruamrungsri, R. Wongpoomchai, Chemical constituents and antioxidant activities of *Cleistocalyx nervosum* fruit in *in vitro* and *in vivo* models, Thai J. Toxicol. 27 (2012) 194–208.
- [13] W. Inboot, S. Taya, A. Chailungka, P. Meepowpan, R. Wongpoomchai, Genotoxicity and antigenotoxicity of the methanol extract of *Cleistocalyx nervosum* var. *Paniala* seed using a Salmonella mutation assay and rat liver micronucleus tests, Mol. Cell. Toxicol. 8 (1) (2012) 19–24.
- [14] B. Portet, N. Fabre, R. Rozenberg, J.L. Habib-Jiwan, C. Moulis, J. Quetin-Leclercq, Analysis of minor flavonoids in *Piper hostmannianum* var. *Berbicense* using liquid chromatography coupled with atmospheric pressure chemical ionization mass spectrometry, J. Chromatogr. A 1210 (1) (2008) 45–54.
- [15] N. Wijit, S. Prasitwattanaseree, S. Mahatheeranont, P. Wolschann, S. Jiranusornkul, P. Nimmanpipug, Estimation of retention time in GC/MS of volatile metabolites in fragrant rice using principle components of molecular descriptors, Anal. Sci. 33 (11) (2017) 1211–1217.
- [16] A. Nilnumkhum, C. Punvittayagul, A. Chariyakornkul, R. Wongpoomchai, Effects of hydrophilic compounds in purple rice husk on AFB1-induced mutagenesis, Mol. Cell. Toxicol. 13 (2) (2017) 171–178.
- [17] C. Punvittayagul, R. Wongpoomchai, S. Taya, W. Pompimon, Effect of pinocembrin isolated from Boesenbergia pandurata on xenobiotic-metabolizing enzymes in rat liver, Drug Metab. Lett. 5 (1) (2011) 1–5.
- [18] P. Sankam, C. Punvittayagul, K. Sringam, C. Chaiyasut, R. Wongpoomchai, Antimutagenicity and anticlastogenicity of glutinous purple rice hull using *in vitro* and *in vivo* testing systems, Mol. Cell. Toxicol. 9 (2) (2013) 169–176.
- [19] A. Chariyakornkul, C. Punvittayagul, S. Taya, R. Wongpoomchai, Inhibitory effect of purple rice husk extract on AFB1-induced micronucleus formation in rat liver through modulation of xenobiotic metabolizing enzymes, BMC Complement. Altern. Med. 19 (1) (2019) 237.
- [20] C. Punvittayagul, A. Chariyakornkul, T. Chewonarin, K. Jarukamjorn, R. Wongpoomchai, Augmentation of diethylnitrosamine-induced early stages of rat hepatocarcinogenesis by 1,2-dimethylhydrazine, Drug Chem. Toxicol. 42 (6) (2019) 641–648.
- [21] R. Puatanachokchai, K. Morimura, H. Wanibuchi, M. Oka, A. Kinoshita, F. Mitsuru, S. Yamaguchi, Y. Funae, S. Fukushima, Alpha-benzene hexachloride exerts hormesis in preneoplastic lesion formation of rat hepatocarcinogenesis with the possible role for hepatic detoxifying enzymes, Cancer Lett. 240 (1) (2006) 102–113.
- [22] T. Thumvijit, S. Taya, C. Punvittayagul, Y. Peerapornpisal, R. Wongpoomchai, Cancer chemopreventive effect of *Spirogyra neglecta*

- A. Chariyakornkul et al.
- Biomedicine & Pharmacotherapy* 133 (2021) 110963
- (Hassall) Kutzing on diethylnitrosamine-induced hepatocarcinogenesis in rats, *Asian Pac. J. Cancer Prev.* 15 (4) (2014) 1611–1616.
- [23] R.P. Adams, Identification of Essential Oil Components by Gas chromatography/ mass Spectrometry, Allured Publishing Corporation, Carol Stream, 2007.
- [24] Y. Liu, T. Yin, Y. Feng, M.M. Cona, G. Huang, J. Liu, S. Song, Y. Jiang, Q. Xia, J. V. Swinnen, G. Bormans, U. Himmelreich, R. Oyen, Y. Ni, Mammalian models of chemically induced primary malignancies exploitable for imaging-based preclinical theragnostic research, *Quant. Imaging Med. Surg.* 5 (5) (2015) 708–729.
- [25] A. Reddig, C.E. Rube, S. Rodiger, P. Schierack, D. Reinhold, D. Roggenbuck, DNA damage assessment and potential applications in laboratory diagnostics and precision medicine, *J. Lab. Precis. Med.* 3 (4) (2018).
- [26] M. Kumar, M. Jain, A. Sehgal, V.L. Sharma, Modulation of CYP1A1, CYP1B1 and DNA adducts level by green and white tea in Balb/c mice, *Food Chem. Toxicol.* 50 (12) (2012) 4375–4381.
- [27] N. Suwannakul, C. Punvittayagul, K. Jarukamjorn, R. Wongpoomchai, Purple rice bran extract attenuates the aflatoxin B1-induced initiation stage of hepatocarcinogenesis by alteration of xenobiotic metabolizing enzymes, *Asian Pac. J. Cancer Prev.* 16 (8) (2015) 3371–3376.
- [28] Y. Shirakami, M.E. Gottesman, W.S. Blaner, Diethylnitrosamine-induced hepatocarcinogenesis is suppressed in lecithin:retinol acyltransferase-deficient mice primarily through retinoid actions immediately after carcinogen administration, *Carcinogenesis* 33 (2) (2012) 268–274.
- [29] H. Jikihara, G. Qi, K. Nozoe, M. Hirokawa, H. Sato, Y. Sugihara, F. Shimamoto, Aged garlic extract inhibits 1,2-dimethylhydrazine-induced colon tumor development by suppressing cell proliferation, *Oncol. Rep.* 33 (3) (2015) 1131–1140.
- [30] T. Rescigno, M.F. Tecce, A. Capasso, Protective and restorative effects of nutrients and phytochemicals, *Open Biochem. J.* 12 (2018) 46–64.
- [31] J. Martel, D.M. Ojcius, Y.F. Ko, P.Y. Ke, C.Y. Wu, H.H. Peng, J.D. Young, Hormetic Effects of phytochemicals on health and longevity, *Trends Endocrinol. Metab.* 30 (6) (2019) 335–346.
- [32] T.G. Jamkhande, P.S. Tidke, Herbal hepatotoxicity: a review on phytochemical induced liver injury, *Int. J. Appl. Pharm. Sci. Res.* 112 (2013) 2471–2479.
- [33] A. Ranjan, S. Ramachandran, N. Gupta, I. Kaushik, S. Wright, S. Srivastava, H. Das, S. Srivastava, S. Prasad, S.K. Srivastava, Role of phytochemicals in cancer prevention, *Int. J. Mol. Sci.* 20 (20) (2019) 4981.
- [34] E.H. Zhang, R.F. Wang, S.Z. Guo, B. Liu, An update on antitumor activity of naturally occurring chalcones, *Evid. Complement. Alternat. Med.* 2013 (2013), 815621.
- [35] H.N. Tuan, B.H. Minh, P.T. Tran, J.H. Lee, H.V. Oanh, Q.M.T. Ngo, Y.N. Nguyen, P. T.K. Lien, M.H. Tran, The Effects of 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone from *Cleistocalyx operculatus* buds on human pancreatic cancer cell lines, *Molecules* 24 (14) (2019) 2538.
- [36] C.L. Ye, Y.F. Lai, 2',4'-Dihydroxy-6'-methoxy-3',5'-dimethylchalcone, from buds of *Cleistocalyx operculatus*, induces apoptosis in human hepatoma SMMC-7721 cells through a reactive oxygen species-dependent mechanism, *Cytotechnology* 68 (2) (2016) 331–341.
- [37] W.G. Yu, J. Qian, Y.H. Lu, Hepatoprotective effects of 2',4'-dihydroxy-6'-methoxy-3',5'-dimethylchalcone on CCl4-induced acute liver injury in mice, *J. Agric. Food Chem.* 59 (24) (2011) 12821–12829.
- [38] H. Ko, Y.J. Kim, E.C. Amor, J.W. Lee, H.C. Kim, H.J. Kim, H.O. Yang, Induction of autophagy by dimethyl cardamonin is associated with proliferative arrest in human colorectal carcinoma HCT116 and LOVO cells, *J. Cell. Biochem.* 112 (9) (2011) 2471–2479.
- [39] R.J. Thoppil, A. Bishayee, Terpenoids as potential chemopreventive and therapeutic agents in liver cancer, *World J. Hepatol.* 3 (9) (2011) 228–249.
- [40] P. Sitarek, P. Rijo, C. Garcia, E. Skala, D. Kalemba, A.J. Bialas, J. Szemraj, D. Pytel, M. Toma, H. Wysokinska, T. Sliwinski, Antibacterial, anti-inflammatory, antioxidant, and antiproliferative properties of essential oils from hairy and normal toots of *Leonurus sibiricus* L. and their chemical composition, *Oxid. Med. Cell. Longev.* 2017 (2017), 7384061.
- [41] M. Chandra, O. Prakash, R. Kumar, R.K. Bachheti, B. Bhushan, M. Kumar, A. Pant, β -Selinene-Rich essential oils from the parts of *Callicarpa macrophylla* and their antioxidant and pharmacological activities, *Medicines* 4 (3) (2017) 52. [42] N.A.A. Ali, B.K. Chhetri, N.S. Dosoky, K. Shari, A.J.A. Al-Fahad, L. Wessjohann, W. N. Setzer, Antimicrobial, antioxidant, and cytotoxic activities of *Ocimum forskolei* and *Teucrium yemense* (Lamiaceae) essential oils, *Medicines* 4 (2) (2017) 17.
- [43] R.A. Popovici, D. Vaduva, I. Pinzaru, C.A. Dehelean, C.G. Farcas, D. Coricovac, C. Danciu, I. Popescu, E. Alexa, V. Lazureanu, H.T. Stanca, A comparative study on the biological activity of essential oil and total hydro-alcoholic extract of *Satureja hortensis* L., *Exp. Ther. Med.* 18 (2) (2019) 932–942.
- [44] R.J. Ogbe, D.O. Ochalefu, S.G. Mafulul, O.B. Olaniru, A review on dietary phytosterols: their occurrence, metabolism and health benefits, *Asian J. Plant Sci. Res.* 5 (2015) 10–21.
- [45] A.C.A. Sanubol, R. Sudmoon, T. Tanee, K. Noikotr, C. Chuachan, Betel-like-scented *Piper* plants as diverse sources of industrial and medicinal aromatic chemicals, *Chiang Mai J. Sci.* 41 (5.1) (2014) 1171–1181.
- [46] A. Chaveerach, S. Aungkapattamagul, T. Tanee, K. Noikotr, R. Sudmoon, Genetic verification and chemical contents identification of *Allamanda* species (Apocynaceae), *Pak. J. Pharm. Sci.* 27 (3) (2014) 417–424.
- [47] M.P. Rahelivao, M. Gruner, H. Andriamanantoanina, B. Andriamihaja, I. Bauer, H. J. Knolker, Red algae (Rhodophyta) from the coast of Madagascar: preliminary bioactivity studies and isolation of natural products, *Mar. Drugs* 13 (7) (2015) 4197–4216.
- [48] S.-H. Chang, J.-H. Bae, D.-P. Hong, K.-D. Choi, S.-C. Kim, E. Her, S.-H. Kim, C.-D. Kang, *Dryopteris crassirhizoma*, *J. Ethnopharmacol.* 130 (2) (2010) 248–254.